Exhibit A

Interval Mapping of *high growth* (*hg*), a Major Locus That Increases Weight Gain in Mice

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ABSTRACT

The *high growth* locus (*hg*) causes a major increase in weight gain and body size in mice. As a first step to map-based cloning of *hg*, we developed a genetic map of the *hg*-containing region using interval mapping of 403 F_2 from a C57BL/6J-*hghg* × CAST/EiJ cross. The maximum likelihood position of *hg* was at the chromosome *10* marker *D10Mit41* (LOD = 24.8) in the F_2 females and 1.5 cM distal to *D10Mit41* (LOD = 9.56) in the F_2 males with corresponding LOD 2 support intervals of 3.7 and 5.4 cM, respectively. The peak LOD scores were significantly higher than the estimated empirical threshold LOD values. The localization of *hg* by interval mapping was supported by a test cross of F_2 mice recombinant between the LOD 2 support interval and the flanking marker. The interval mapping and test-cross results indicate that *hg* is not allelic with candidate genes *Igf1* or *decorin* (*Dcn*), a gene that was mapped close to *hg* in this study. The *hg* inheritance was recessive in females, although we could not reject recessive or additive inheritance in males. Possible causes for sex differences in peak LOD scores and for the distortion of transmission ratios observed in F_2 males are discussed. The genetic map of the *hg* region will facilitate further fine mapping and cloning of *hg*, and allow searches for a homologous quantitative trait locus affecting growth in humans and domestic animals.

THE *high growth* locus (*hg*) is a major locus that increases weight gain and mature body size of mice by 30–50% and was originally described as a recessive gene with nearly complete penetrance (BRADFORD and FAMULA 1984). There is now evidence that *hg* can be partially expressed in male heterozygotes (MEDRANO *et al.* 1992). The *hg* locus increases the efficiency of growth by influencing energy metabolism (CALVERT *et al.* 1986) without altering overall body composition (CALVERT *et al.* 1985). Cloning of the *hg* locus would permit functional characterization of *hg* and enable studies of homologous loci in humans and domestic animal species. In the latter, this may lead to identification of a quantitative trait locus (QTL) for rapid and efficient growth, a trait of significant economic importance in the livestock industry.

Previous genetic analyses (MEDRANO *et al.* 1992) established linkages between *hg* and *insulin-like growth factor-1* (*Igf1*) and *Steel* (*Sl*), two loci on mouse chromosome *10*. A concurrent physiological study (MEDRANO *et al.* 1991) determined that the inheritance of *hg* is associated with elevated circulating levels of IGF1, a finding that suggested *Igf1* as a candidate gene for *hg*. To test the hypothesis that *hg* is allelic with *Igf1* and as a prerequisite for physical cloning of *hg*, a finer genetic map of the *hg*-containing region was needed.

One approach for improving the map resolution of a locus affecting a quantitative trait is by interval mapping (LANDER and BOTSTEIN 1989) in larger crosses between genetically distant inbred lines using dense molecular genetic maps. This approach has been used to identify map locations of QTLs in plants (PATERSON *et al.* 1988, 1991; STUBER *et al.* 1992) and in mammals (JACOB *et al.* 1991).

This study was designed to develop a genetic map of the region around *hg* and to characterize *hg* inheritance and phenotypic effects using interval mapping in a large F_2 cross between C57BL/6J-*hghg* (*Mus musculus domesticus*) and CAST/EiJ mice (*M. musculus castaneus*). In a search for other potential candidates from the homologous region in humans, we developed and mapped a genetic marker for the murine *decorin* (*Dcn*) gene. A test cross of selected F_2 recombinant mice was performed to verify the localization of *hg* determined by interval mapping, and/or to test for allelism between *hg* and *Igf1* and between *hg* and *Dcn*.

MATERIALS AND METHODS

Mapping population: A thorough description of the discovery of *hg* is presented in BRADFORD and FAMULA (1984). The *hg* locus has since been introgressed into the C57BL/6J background by nine backcrosses to create congenic mice C57BL/6J-*hghg*. In this experiment, animals from the sixth generation of inbreeding C57BL/6J-*hghg* mice (HG) were used. To initiate our mapping cross, eight HG females were crossed to two CAST males (CAST/EiJ line; Jackson Laboratory, Bar Harbor, ME). A total of 62 F_1 mice were produced. The F_2 generation (403 mice) was generated by 42 brother-sister F_1 matings; there were 13, 14 and 4 F_2 litters of the first, second

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and third parity matings, respectively. The F_2 mice were weighed weekly from 14 to 63 days of age.

Genotyping: All the mice from the parental, F_1 and F_2 generations were genotyped for nine microsatellite markers: *D10Mit9*, *D10Mit10*, *D10Mit12*, *D10Mit14*, *D10Mit31*, *D10Mit41*, *D10Mit42* (Dietrich *et al.* 1992), *D10Nds2* (Cornall *et al.* 1991) and *Igf1* (Medrano *et al.* 1992). F_2 genotype data (*n* = 103) was submitted to Mouse Genome Database (accession number MGD-CREX-234). *Igf1* microsatellite primers used were IGF3 (5′-AGTCGAGGGATTTGAATGAG-ATCAT) and IGF8 (5′-TGTCACCTCCTCCACCCCAAGGG). Markers *D10Mit9*, *D10Mit10*, *D10Mit12*, *D10Mit14*, *D10Mit42* and *Igf1* were assayed using a polymerase chain reaction (PCR) essentially as described in Dietrich *et al.* (1992); for *D10Mit42* the annealing temperature was increased to 60° and the number of cycles to 40. Primers were obtained from Research Genetics (Huntsville, AL). PCR reactions were run in flexible, 96 U-bottom well plates (FALCON 3911, Becton Dickinson, Oxnard, CA) on Thermal Cycler PTC96 (MJ Research, Watertown, MA). To increase the efficiency of genotyping, some markers were amplified in the same PCR reaction (multiplexed) and/or loaded together on the same 7% denaturing polyacrylamide gel as follows: a multiplex PCR reaction of *D10Mit9*, *D10Mit10*, *D10Mit14* was analyzed on the same gel; a multiplex PCR reaction of *D10Mit42* and *Igf1* was loaded together with *D10Mit42*. Markers *D10Nds2*, *D10Mit31* and *D10Mit41* were amplified following the conditions described for *D10Nds2* (Cornall *et al.* 1991) and PCR products were analyzed on 2.5% agarose gels (IBI, New Haven, CT). Autoradiograms and pictures of agarose gels were independently scored twice by two people. Markers *D10Mit42*, *D10Mit9*, *D10Mit10*, *D10Mit41*, *D10Mit12* and *D10Nds2* were chosen to cover the genomic region between *Igf1* and *Sted* (*Sd*), which was considered a candidate region for *hg* from previous mapping studies (Medrano *et al.* 1992). The proximal marker *D10Mit31* was included because of its linkage to oncogenes (*e.g.*, *Fyn*, *Ras1*) and a *cell division cycle 2 homolog* (*Cdc2a*), genes involved in the control of cell differentiation and growth. The distal marker, *D10Mit14*, was studied because of its linkage to *pygmy* (*pg*), a locus with a known mutation affecting body size (MacArthur 1944).

Generation of a PCR-based marker for the murine *Dcn* gene and genetic mapping: We searched for polymorphisms between HG and CAST DNAs in intron 4 of the murine *Dcn* gene. The intron 4 sequences were first amplified with primers in exon 4 (DCNE4P5 = 5′-TGTCCATGAGAATGAGATCACCA) and in exon 5 (DCNE5P3 = 5′-GGCTTCCAGGGACTGAAGAGTCT). These two primers were designed on the basis of a mouse cDNA decorin sequence (EMBL accession No. X53929) and generated a 1.1-kb PCR product. The DCNE4P5–DCNE5P3 PCR product was sequenced from both ends using an *f*mol-Cycle Sequencing Kit (Promega, Madison, WI). Upon identification of a *Tpi* polymorphic site 85 bp downstream of exon 4, another primer was designed in intron 4 (DCNE4P3 = 5′-CTGGGCACAAGCACCTTGTCAC). Primers DCNE4P5 and DCNE4P3 amplified a 240-bp fragment (GenBank accession No. L38611) spanning the *Tpi* polymorphic site. In the HG DNA, two *Tpi* sites in the DCNE4P5–DCNE4P3 PCR product produced fragments of 25, 84 and 131 bp; in the CAST DNA, one *Tpi* site produced two fragments of 131 and 109 bp (Figure 1). The PCR-amplification components for DCNE4P5–DCNE4P3 product were 40 ng DNA, 0.3 μM of each primer, 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH = 9.0, 0.1% Triton X-100, Promega), 150 μM each of dNTPs and 2 mM $MgCl_2$, 0.5 units of *Taq* DNA polymerase (Promega) in a final volume of 20 μl. The amplification profile was 30 cycles at 94° (45 sec), 60° (1 min) and 72° (1 min); in the first cycle the denaturation step was 97° (2 min) and in the last cycle, the extension time at 72° was increased to 5 min. PCR products were digested for 4 hr at 65° by directly adding to the PCR reaction (under oil) a premix containing 1 unit *Tpi* (New England Biolabs, Beverly, MA), 3.0 μl of 10× buffer 3 and 6.5 μl sterile water. Digests were analyzed by electrophoresis in 3% 0.5× TBE-agarose. The *Dcn* marker was first mapped to the interval between *D10Mit41* and *D10Mit12* using a subset of informative recombinant F_2 mice. The genetic distances from *Dcn* to *D10Mit41* and *D10Mit12* were then determined using 28 F_2 mice (17 females, 11 males), that were recombinant for the *D10Mit41* to *D10Mit12* interval. We also tested this marker in six standard inbred strains. Strains A/J, C57BL/6J, DBA/2J and C3H/HeJ produced a pattern observed in HG mice and strains AKR/J and SPRET/EiJ produced a pattern of CAST.

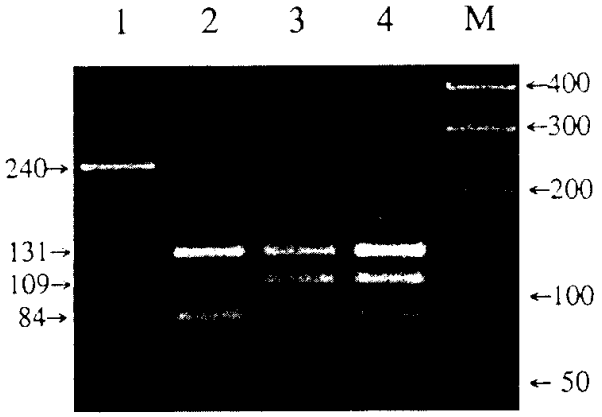

Figure 1.—A *Tpi* PCR-RFLP marker in the murine *decorin* (*Dcn*) gene with fragment sizes (in base pairs) displayed on the left side: Lane 1, undigested PCR product; lane 2, HG (C57BL/6J-*hg*/*hg*) DNA; lane 3, CAST (CAST/EiJ) DNA; lane 4, F_1; lane M, DNA size marker (50- to 2000-bp size marker, BIO-RAD, CA) with sizes displayed on the right side. Standard inbred strains C57BL/6J, DBA/2J, A/J and C3H/HeJ show pattern of lane 2, while AKR/J and SPRET/EiJ that of lane 3.

Test cross: The test cross involved matings between F_1 and HG mice. Mice were weighed at 21 and 42 days of age.

To test for allelism between *hg* and *Igf1*, five types of crosses were performed (Tables 1 and 2). A first control cross involved F_2 mice (3 females and 3 males) that were heterozygous for both *Igf1* and markers spanning the *hg* LOD 2 support interval (*D10Mit9*, *D10Mit10*, *D10Mit14*, *D10Mit12*). This cross was performed to test for *hg* segregation and/or significant weight gain differences between progeny homozygous for HG alleles at *Igf1* and *hg* LOD 2 support interval and heterozygous progeny (cross I, Tables 1 and 2). A second control cross included three F_2 females and two F_2 males that were homozygous for CAST alleles for *Igf1* and *hg* LOD 2 support interval (cross II, Tables 1 and 2). Crosses of recombinant F_2 mice involved one female heterozygous at *Igf1* and homozygous for CAST alleles in the *hg* LOD 2 support interval (cross III, Tables 1 and 2), two males heterozygous at *Igf1* and homozygous for HG alleles in the *hg* LOD 2 support interval (cross IV, Tables 1 and 2) and two females and two males homozygous for HG alleles at *Igf1* and heterozygous in the *hg* LOD 2 support interval (cross V, Tables 1 and 2). Progeny of these crosses were typed for *Igf1* and *D10Mit41* as described above. Typings at *D10Mit41* were considered to represent the genotypes within the *hg* LOD 2 support interval.

To test for allelism between *hg* and *Dcn*, we test crossed an F_1 female that was heterozygous at *Igf1*, *D10Mit9*, *D10Mit10*,

TABLE 1

Test of allelism between *Igf1* and *hg*: female progeny from a F_2 × HG test cross

Cross[a]	Genotype of F_2 parents		Genotype of female progeny			
			Igf1: HG/HG "*hg*": HG/HG	HG/CAST HG/CAST	HG/HG HG/CAST	HG/CAST HG/HG
I	*Igf1*:	HG/CAST	11.0 ± 2.9*	8.1 ± 1.6*	8.3	12.3
	"*hg*":	HG/CAST	(13)	(21)	(1)	(1)
II	*Igf1*:	CAST/CAST		7.1 ± 1.2		
	"*hg*":	CAST/CAST		(15)		
III	*Igf1*:	HG/CAST		8.4 ± 1.8	8.1 ± 1.1	
	"*hg*":	CAST/CAST		(9)	(10)	
IV	*Igf1*:	HG/CAST	13.3 ± 1.8			14.1 ± 1.8
	"*hg*":	HG/HG	(3)			(12)
V	*Igf1*:	HG/HG	11.2 ± 2.3*		7.9 ± 1.6*	
	"*hg*":	HG/CAST	(17)		(15)	

Mean of weight gain from 21 to 42 days of age (g) ± SD (number of animals in parentheses). Means of progeny from a particular cross (*i.e.*, within a row) marked with * differ significantly (*t*-test, $P < 0.01$). HG, C57BL/6J-*hghg* alleles; CAST, CAST/EiJ alleles; "*hg*", markers defining the *hg* LOD 2 support interval (*D10Mit9, D10Mit10, D10Mit41, D10Mit12*).

[a] In each cross, progeny were pooled from several test crossed F_2 mice (see MATERIALS AND METHODS).

D10Mit41 and homozygous for HG alleles at *Dcn*. This female produced a total of 35 progeny in four parities. Progeny were typed for *D10Mit41* and *Dcn*.

Data analyses: Weight gain from 14 to 63 days of age in F_2 mice was analyzed by least-squares using procedure GLM of SAS (1985), with a model incorporating an overall mean, fixed effects of sex, litter size, parity, all two- and three-way interactions and a random residual error. Only the main effect of sex was found to be significant ($P < 0.001$). Therefore, the interval mapping and all other analyses were done, on unadjusted data, separately for each sex. Although we could have made statistical adjustment for the effect of sex and pooled the female and male data sets, analyses separated by sex were more conservative and revealed new information that could have been hidden in pooled data. For example, interval analysis showed that the mode of inheritance of *hg* and the magnitude of peak LOD scores differed between the sexes, and transmission distortion of chromosome *10* markers was uncovered in F_2 males.

Linkage analyses were performed on PC using MAPMAKER/EXP 3.0 software (LANDER *et al.* 1987; LINCOLN *et al.* 1992a). The map position of *hg* was determined by interval mapping (LANDER and BOTSTEIN 1989) using MAPMAKER/QTL 1.1 software (PATERSON *et al.* 1988; LINCOLN *at al.* 1992b). To correct for nonnormality of the distribution of the weight gain variable, we used a logarithmic transformation of weight gain from 14 to 63 days of age in the MAPMAKER/QTL analysis. The inheritance of *hg* was examined by comparisons of the peak LOD scores obtained by unconstrained, recessive ($d = -a$), additive ($d = 0$) and dominant ($d = a$) F_2 trait models. Detailed explanations of F_2 trait models can be found in LINCOLN *et al.* (1992b) and PATERSON *et al.* (1991). If the peak LOD scores computed under the recessive, additive and dominant models deviated from the uncon-

TABLE 2

Test of allelism between *Igf1* and *hg*: male progeny from a F_2 × HG test cross

Cross[a]	Genotype of F_2 parents		Genotype of male progeny			
			Igf1: HG/HG "*hg*": HG/HG	HG/CAST HG/CAST	HG/HG HG/CAST	HG/CAST HG/HG
I	*Igf1*:	HG/CAST	18.0 ± 2.8*	13.3 ± 2.6*	12.3	18.3
	"*hg*":	HG/CAST	(20)	(22)	(1)	(1)
II	*Igf1*:	CAST/CAST		12.4 ± 1.9		
	"*hg*":	CAST/CAST		(17)		
III	*Igf1*:	HG/CAST		12.9 ± 2.5	11.6 ± 2.8	
	"*hg*":	CAST/CAST		(20)	(22)	
IV	*Igf1*:	HG/CAST	22.6 ± 2.1			24.3 ± 2.6
	"*hg*":	HG/HG	(4)			(3)
V	*Igf1*:	HG/HG	19.4 ± 4.0*		13.4 ± 3.2*	
	"*hg*":	HG/CAST	(18)		(24)	

Mean of weight gain from 21 to 42 days of age (g) ± SD (number of animals in parentheses). Means of progeny from a particular cross (*i.e.*, within a row) marked with * differ significantly (*t*-test, $P < 0.01$). HG, C57BL/6J-*hghg* alleles; CAST, CAST/EiJ alleles; "*hg*", markers defining the *hg* LOD 2 support interval (*D10Mit9, D10Mit10, D10Mit41, D10Mit12*).

[a] In each cross, progeny were pooled from several test crossed F_2 mice (see MATERIALS AND METHODS).

strained model by more than two LOD score units, which corresponds to at least a 100-fold reduction in the likelihood ratio, the model was not considered as a likely inheritance model for *hg*. Similarly, regions outside the *hg* LOD 2 support interval (*i.e.*, regions that yielded LOD scores lower by at least two units compared with the LOD score at the *hg* maximum likelihood position) were not considered as regions likely to contain *hg*. SAS software (SAS 1985) was used to compute frequencies and probabilities of the chi-square values in the marker segregation analyses, and to compare means (*t*-test) between genotypic classes in F_2 and test-cross progeny.

Because the peak LOD scores at the *hg* maximum likelihood location were lower in F_2 males (LOD = 9.56) than F_2 females (LOD = 24.8), we examined whether the effects of *hg* between the sexes could account for the peak LOD scores differences. The difference between the mean weight gain from 14 to 63 days of age of HG/HG and CAST/CAST genotypes at *D10Mit41* (marker closest to the *hg* maximum likelihood location) were 5.2 g and 4.5 g in F_2 females and males, respectively. The means of the HG/CAST and CAST/CAST genotypes differed by 0.8 g and 1.5 g in F_2 females and males, respectively. On the basis of these differences, we adjusted the weight gain from 14 to 63 days of age in the male data to the magnitude of the effect in F_2 females, by increasing the weight gain in HG/HG males by 0.7 g and decreasing it in HG/CAST males by 0.7 g. The modified male data set was then analyzed using MAPMAKER/QTL 1.1 software package as described above (see DISCUSSION).

Estimation of empirical LOD score threshold values: Empirical threshold values were estimated for the F_2 females and males as described by CHURCHILL and DOERGE (1994). The original set of trait values (logarithm of weight gain from 14 to 63 days of age) was randomly permuted (shuffled) to generate 1000 shuffled data sets. A shuffled data set was generated by indexing the original set of trait values, assigning a random number to this trait-index, and sorting the traits by random numbers. This procedure was repeated 1000 times. The shuffled data sets along with the original (unshuffled) set were then analyzed by MAPMAKER/QTL software version 0.9 for VAX/VMS. The experimentwise critical value was obtained by ordering the maximum LOD scores from each of the 1000 analyses of shuffled data sets and locating their 950th ($1 - \alpha = 0.95$) and 990th ($1 - \alpha = 0.99$) value. The programs for shuffling and other manipulation of data were made using SAS software (1985).

RESULTS

Interval mapping of the *hg* locus: A genetic map of the *hg*-containing region in the HG × CAST intercross is displayed in Figure 2. The *hg* locus was localized by interval mapping using genetic markers from the distal half of the mouse chromosome *10*, and the trait, logarithm of weight gain from 14 to 63 days of age. The maximum likelihood position of *hg* in the F_2 females was at *D10Mit41* (Figure 2A, LOD = 24.81) and in the F_2 males 1.5 cM distal to *D10Mit41* (Figure 2B, LOD = 9.56). This location accounted for 41.5 and 22.2% of the F_2 variance in females and males, respectively. The LOD 2 support interval for the *hg* location encompassed a 3.7-cM (females) and a 5.4-cM (males) region surrounding the corresponding maximum likelihood positions. The male and female LOD 2 support intervals overlap in the interval of 2.6 cM from the position 0.6 cM proximal to *D10Mit41* to the position 2 cM distal to *D10Mit41*.

LOD score threshold values: The peak LOD scores in our analyses appeared to be highly significant when compared with "typical" threshold values of between 2 to 3 (for $\alpha = 0.05$) suggested by LANDER and BOTSTEIN (1989). However, these threshold values may not be appropriate for use in our study, because our experimental conditions were different from the conditions assumed in LANDER and BOTSTEIN's study. Our QTL analysis was based on the markers defining a single linkage group of ~30 cM rather than on markers covering the whole genome. Also, in the males, segregation distortion was encountered. Differences in these and other experimental conditions may require different threshold LOD values for detecting significant QTL effects (CHURCHILL and DOERGE 1994). We hence determined the empirical threshold values that applied to characteristics of our experiment (see MATERIALS AND METHODS). The empirical experimentwise threshold LOD scores for the significance level of $\alpha = 0.05$ were 1.95 and 1.97 in F_2 females and males, respectively, and for the significance level of $\alpha = 0.01$ were 2.7 and 2.95 in F_2 females and males, respectively. The observed peak LOD scores were much larger than the estimated empirical threshold LOD scores indicating a significant effect of *hg*.

Inheritance and phenotypic effects of *hg*: To investigate the mode of inheritance of *hg*, LOD scores computed by the unconstrained F_2 trait model were compared to LOD scores obtained by constrained models such as pure recessive, pure dominant, and pure additive (Figure 2). If the likelihood of a constrained model showed a deviation of 2 LOD units or more from unconstrained model likelihoods, then we considered that type of gene action unlikely. We could not reject recessive inheritance for females and additive or recessive inheritance for males. The mode of inheritance and phenotypic effects of *hg* in this cross is also observed comparing the mean weight gains of the three F_2 genotypic classes at the marker *D10Mit41* (Figure 3). HG/HG F_2 females and males were significantly larger (*t*-test, $P < 0.01$) than HG/CAST and CAST/CAST mice. HG/CAST F_2 males were also significantly larger than CAST/CAST males, but this was not the case with females. These results confirm the analysis of *hg* inheritance by interval mapping indicating a recessive mode of action in F_2 females and a partially recessive mode in F_2 males.

Close linkage of *hg* and *Dcn*: Many loci from the distal half of mouse chromosome *10* belong to a block of homologous genes on human chromosome *12 q13-q24* (COPELAND *et al.* 1993; O'BRIEN *et al.* 1993). Hence, a human homolog of *hg* is expected to reside in that region. We noted that the human decorin (DCN) gene, a ubiquitous interstitial proteoglycan shown to be involved in cell proliferation and extracellular matrix as-

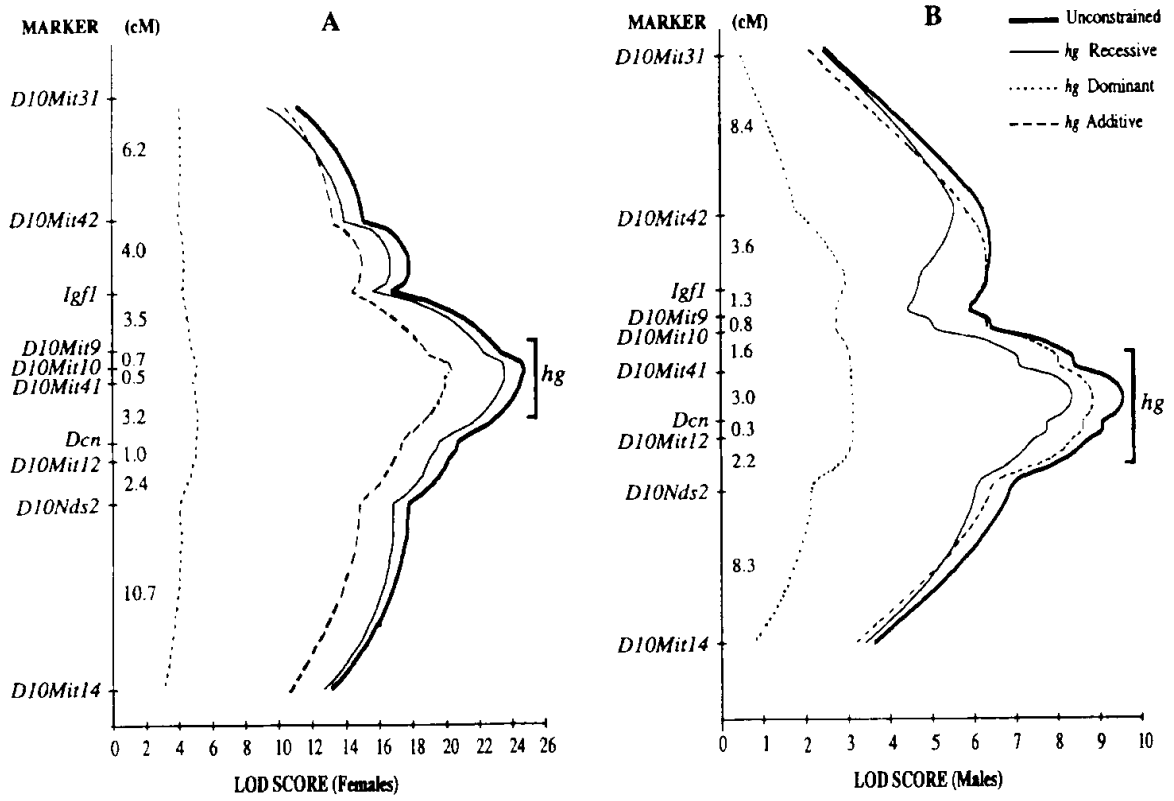

FIGURE 2.—Interval mapping of the *high growth* locus (*hg*) using the logarithm of weight gain from 14 to 63 days of age (A, based on F_2 female data; B, based on F_2 male data). QTL maximum likelihood plots for the four F_2 inheritance models (unconstrained, recessive, additive, dominant) are displayed. The bracket indicates the LOD 2.0 support interval for the maximum likelihood location of *hg*.

sembly control (RUOSLAHTI and YAMAGUCHI 1991), maps to human chromosome *12q23* (DANIELSON *et al.* 1993) or *12q21.3.* (VETTER *et al.* 1993). Therefore, we examined whether the murine *Dcn* gene maps to the distal half of mouse chromosome *10* and if so, how close to the *hg* locus. We developed a PCR-based marker for the murine *Dcn* gene (Figure 1), and mapped it 3 cM distal to *D10Mit41* (Figure 2). In relation to *hg*, the *Dcn* gene maps within the *hg* LOD 2 support interval in males and falls 1.2 cM beyond the distal border of the *hg* LOD 2 support interval in females. The *hg* locus, therefore, appears to be closely linked to the murine *Dcn* gene.

Test crosses to verify the localization of *hg* obtained by interval mapping and test for allelism between *hg* and *Igf1* and between *hg* and *Dcn*: Our mapping analysis (Figure 2) positioned the *Igf1* locus ~3 cM proximal to the boundary of the *hg* LOD 2 support interval. LOD scores for *hg* at *Igf1* were 8 units (females) or 3.3 units (males) lower than the maximum likelihood LOD scores. These results suggest that *hg* and *Igf1* are not allelic. To provide further support for this hypothesis and to verify if *hg* is located distal to *Igf1* as implied from interval mapping, F_2 mice, recombinant and nonrecombinant between the *Igf1* locus and the *hg* LOD 2 support interval, were crossed with HG mice. The progeny of nonrecombinant F_2 mice that were heterozygous for both *Igf1* and *hg* LOD 2 support interval (cross I, Tables 1 and 2) segregated two significantly different weight gain phenotypes between mice carrying HG alleles at *Igf1* and *hg* LOD 2 support interval and heterozygotes. The progeny of F_2 mice that were heterozygous for the *hg* LOD 2 support interval but were homozygous for HG alleles at *Igf1* (cross V, Tables 1 and 2) also showed a similar segregation, in that progeny homozygous for HG alleles within the *hg* LOD 2 support interval were significantly larger compared to littermates heterozygous for the *hg* LOD 2 support interval. In contrast, progeny of F_2 mice heterozygous at *Igf1* but homozygous within the *hg* LOD 2 support interval for CAST alleles (cross III, Tables 1 and 2) or HG alleles (cross IV, Tables 1 and 2) did not exhibit segregation and the means for weight gain from 21 to 42 days of age of heterozygous and homozygous progeny for HG alleles at *Igf1* did not differ significantly. These results support the implication from interval mapping that *hg* and *Igf1* are not allelic, and that *hg* is located distally to *Igf1*.

To verify the *hg* location in relation to the distal border of the *hg* LOD 2 support interval, and/or to test for allelism between *hg* and *Dcn*, a test cross of an F_3 female heterozygous at *Igf1, D10Mit9, D10Mit10, D10Mit41* and homozygous for HG alleles at *Dcn* was

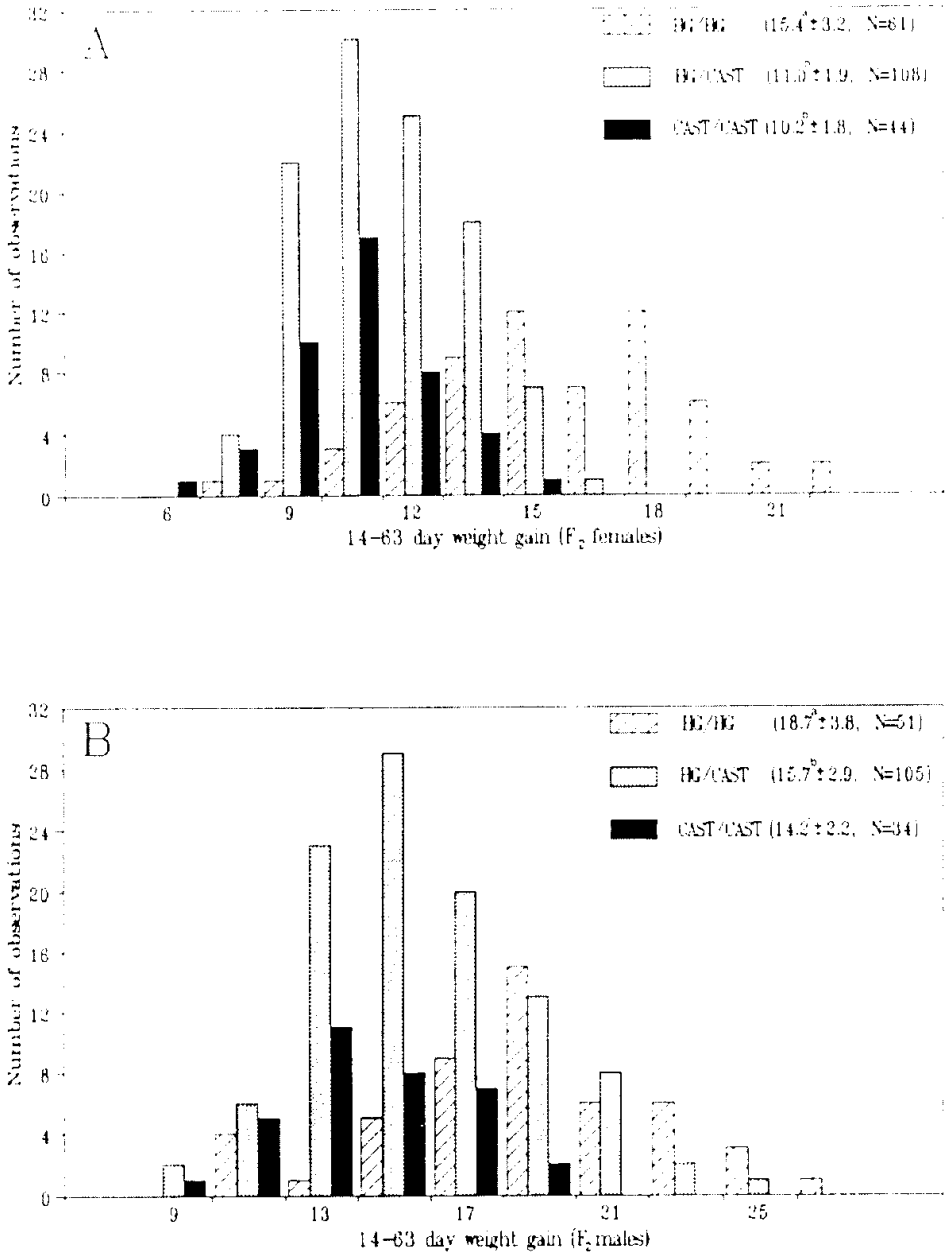

FIGURE 3.—Distribution of number of observations by genotypic classes at the *D10Mit41* locus for the weight gain from 14 to 63 days of age in F_2 females (A) and F_2 males (B); HG, CAST indicate alleles from C57BL/6J-*hghg*, CAST/EiJ parents, respectively. The numbers in parentheses indicate the means ± SD and a number of individuals (n) of a particular genotypic class. Means of the genotypic classes marked with different superscripts (a, b, c) differ significantly (t-test, $P < 0.01$). The overall mean ± SD of the F_2 population was 12.1 ± 3.17 (n = 213) and 16.2 g ± 3.47 (n = 190) in females and males, respectively.

performed. If *hg* were an allele of *Dcn*, all the progeny would be expected to have a high-growth phenotype. Conversely, if *hg* localized within the *hg* LOD 2 support interval, progeny would be segregating for *hg*. The mean ± SD for weight gain from 21 to 42 days of age of the progeny homozygous for HG alleles at *D10Mit41* and *Dcn* was 12.4 ± 1.7 g for females (n = 7) and 20.9 ± 3.6 g for males (n = 12). These weight gains were significantly higher (t-test, $P < 0.05$) than the weight gains of their sisters (10.3 ± 1.4 g, n = 10) or their brothers (15.7 ± 2.0 g, n = 6) who were heterozygous at *D10Mit41* but homozygous for HG alleles at *Dcn*, suggesting a segregation for *hg*. Therefore, the *hg* locus is not allelic with *Dcn* and resides proximally to the murine *Dcn* gene as implied from the peak LOD scores from interval analysis.

Segregation of markers: To test if the segregation of markers was consistent with the expected Mendelian

TABLE 3

Genotype frequencies and chi-square probabilities of allelic and genotypic segregation in the HG × CAST intercross

	F_2 females (n = 213)					F_2 males (n = 190)				
	Genotype frequencies (%)			Chi-square probabilities[a]		Genotype frequencies (%)			Chi-square probabilities[a]	
Locus	HG/HG	HG/CAST	CAST/CAST	Genotypes	Alleles	HG/HG	HG/CAST	CAST/CAST	Genotypes	Alleles
D10Mit31	25.8	54.9	19.3	0.14	0.17	28.4	53.7	17.9	0.07	0.04
D10Mit42	27.2	52.6	20.2	0.27	0.15	27.9	57.3	14.8	0.01	0.01
Igf1	26.7	50.2	21.1	0.42	0.24	27.4	57.3	15.3	0.01	0.02
D10Mit9	28.2	50.2	21.6	0.40	0.18	26.3	57.9	15.8	0.01	0.04
D10Mit10	28.6	50.7	20.7	0.25	0.10	26.3	57.4	16.3	0.02	0.05
D10Mit41	28.6	50.7	20.7	0.25	0.10	26.8	55.3	17.9	0.07	0.08
D10Mit12	27.7	49.3	23.0	0.61	0.33	27.4	55.3	17.3	0.05	0.05
D10Nds2	27.2	51.2	21.6	0.48	0.24	26.8	56.3	16.9	0.03	0.05
D10Mit14	28.2	47.9	23.9	0.56	0.38	30.5	51.1	18.4	0.06	0.02

[a] Expected Mendelian ratios: genotypes 1:2:1, alleles 1:1.

ratios of 1:2:1 for genotypic frequencies and 1:1 for allele frequencies, a chi-square analysis was performed for 213 F_2 females and 190 F_2 males. In the males, significant deviations ($P \leq 0.05$) from the expected genotypic and allele frequencies were observed for most markers (Table 3). The main contributing factor to distortion of transmission ratios in F_2 males appears to be an underrepresentation of homozygotes for the CAST alleles at chromosome *10* markers analyzed here. In F_2 females, however, no significant deviations from expected genotype and allele segregation ratios were observed. We noted, though, that a proportion of CAST/CAST homozygotes in F_2 females also tended to be lower, although not as pronounced as in F_2 males.

DISCUSSION

Interval mapping of *hg* and test crossing: We developed a genetic map of a region containing *hg* by interval mapping analysis in a large HG × CAST F_2 cross. The maximum likelihood position of *hg* was at *D10Mit41* (LOD = 24.8) in F_2 females and 1.5 cM distally from that locus in F_2 males (LOD = 9.56) with the surrounding 3.7-cM (females) and 5.4-cM (males) LOD 2 support interval. The interval mapping thus yielded similar maximum likelihood positions between sexes with highly significant LOD scores, and narrow LOD 2 support intervals. Inheritance of *hg* was essentially recessive in F_2 females. In F_2 males, however, results demonstrate partial recessiveness of *hg* suggesting some expression of *hg* in male heterozygotes and confirming our observations from previous crosses (MEDRANO *et al.* 1992).

The genetic mapping of *hg* obtained in the present study is supported by previous linkage studies (MEDRANO *et al.* 1992) that suggested a putative location of *hg* between two loci on mouse chromosome *10*, *Igf1* and *Steel*. This region of the mouse chromosome *10* has also been reported to contain QTL(s) that increase growth in a population of Quackenbush-Swiss (QS) mice (COLLINS *et al.* 1993). Strong associations were found between *D10Mit12* and *D10Mit14* genotypes and increased 42-day body weight. It would be of interest to determine whether the enhancing effect on growth observed in *hg* and QS mice is controlled by an allele(s) at the *hg* locus or different linked loci.

The results of the test-cross experiment provided further support for the localization of *hg* by interval mapping, and also suggested that *hg* is not allelic with *Igf1* and *Dcn*. The *Igf1* gene was considered a possible candidate for *hg* because of genetic linkage (MEDRANO *et al.* 1992) and because elevated levels of IGF1 plasma protein were detected in high growth mice (MEDRANO *et al.* 1991). However, in the present interval mapping analysis, the *Igf1* locus was localized ~3 cM outside the proximal border of the *hg* LOD 2 support interval. A test cross of F_2 mice recombinant between the *Igf1* locus and the *hg* LOD 2 support interval revealed that the test-cross progeny homozygous for HG alleles within the *hg* LOD 2 support interval expressed the high-growth phenotype regardless of their allelic composition at the *Igf1* locus, whereas the test-cross progeny homozygous for the HG alleles at *Igf1* but heterozygous for the *hg* LOD 2 support interval did not express the high-growth phenotype. These results demonstrate that *Igf1* and *hg* are two separate loci and that *hg* is located distally from the *Igf1* locus, as suggested by the peak of the LOD-QTL curve and its LOD 2 support interval. The localization of *hg* to the *hg* LOD 2 support interval was also confirmed in a test cross of an F_3 female recombinant between the *Dcn* and markers in the *hg* LOD 2 support interval. The progeny that were homozygous for HG alleles within the *hg* LOD 2 support interval and *Dcn* expressed the high-growth phenotype while their littermates that were heterozygous for *hg* LOD 2 support interval and homozygous for HG alleles at *Dcn* did not express the high-growth phenotype. This result suggests

that *hg* is located proximally to *Dcn*. The combination of the *hg* interval map and the test-cross results provide genetic evidence that *hg* is not an allele of *Igf1* or *Dcn*, and that the genomic segment between *D10Mit9* and *Dcn* is likely to contain the *hg* locus.

The high-growth mice appeared in a stock subjected to selection for rapid postweaning growth (BRADFORD and FAMULA 1984). Because the high-growth mice appeared in a single litter in one of the two closely related sublines, it was suggested that the origin of *hg* was most likely a spontaneous mutation in a single gene. The authors also noted that *hg* could be a preexisting allele whose effects could be uncovered in a background of positive growth QTLs generated by selection. Another possible explanation for the origin of *hg* could be that recombination produced a desirable combination of two (or more) closely linked alleles that would behave genetically as a single *hg* locus. Common to these explanations is the assumption that *hg* resides in a single locus. This assumption is supported by the present study in that interval mapping revealed the *hg* location with a defined peak of a QTL curve and narrow *hg* LOD 2 support interval. Furthermore, the positioning of *hg* in the LOD 2 support interval was verified by a test-cross experiment. However, because the interval mapping method can not resolve closely linked QTLs, we cannot eliminate the possibility that the *hg* effect is controlled by two (or more) alleles, linked within the *hg* LOD 2 support interval. Future fine resolution *hg* mapping and cloning should reveal whether *hg* is a single allele or a cluster of linked alleles.

Threshold LOD scores and sex differences in the *hg* peak LOD scores: The estimated empirical threshold LOD score values were essentially the same in the F_2 females and males and similar to threshold values typically used to declare a significance of a QTL effect (LANDER and BOTSTEIN 1989). This suggests that the specific features of our experiment (*e.g.*, only a single linkage group used and transmission distortion present in the males), did not markedly influence the threshold LOD values. The 1000 shuffled data-sets used to estimate the empirical threshold LOD values in this study will be utilized in our future QTL analyses of this cross to determine threshold LOD values for growth QTLs located elsewhere on the genome. Such empirical threshold LOD estimations will be particularly useful in assessing the significance of effects of QTLs with small effects.

A large difference in the LOD scores at the maximum likelihood position of *hg* was detected between the sexes (24.8 and 9.56 LOD units in females and males, respectively). Some possible causes for this may be differences in the allelic and dominance effect of *hg* and/or differences in epistatic effects of growth QTLs between the sexes, differences in the number of growth QTLs between the sexes and differences in the genetic composition of the *X* chromosome(s) between our F_2 females and F_2 males which may have generated differences in the effects of assumed *X*-linked growth QTLs. These factors may have decreased the overall importance of the effect of *hg* on the total genetic or phenotypic variation of F_2 males, which in turn could be responsible for the observed lower LOD scores in F_2 males.

Sexual dimorphism in the allelic and epistatic effects has been demonstrated for bristle genetic factors in Drosophila (LONG *et al.* 1995). In our cross, the standardized allelic effect, $^1/_2\{[\bar{X}_{HG/HG} - \bar{X}_{CAST/CAST}]/SD_{F2\delta or \varphi}\}$, at *D10Mit41* was 0.82 in F_2 females and 0.65 in F_2 males suggesting a slightly lower effect of *hg* in F_2 males. Also, the analysis of different inheritance models in interval mapping and the comparison of standardized dominance effects, $\{[\bar{X}_{HG/CAST} - {}^1/_2(\bar{X}_{HG/HG} + \bar{X}_{CAST/CAST})]/SD_{F2\delta or \varphi}\}$, at *D10Mit41* (−0.57 and −0.22 in F_2 females and F_2 males, respectively) indicate sex differences in the dominance effect of *hg*. We thus examined how the peak LOD score in F_2 males would change if we made the effect of *D10Mit41* in F_2 males similar to F_2 females (see MATERIALS AND METHODS). The MAPMAKER/QTL analysis of the modified F_2 male data set yielded a peak LOD score of 14.4 units. This increase of 4.8 LOD units in comparison with the peak LOD score (LOD = 9.56) of the original F_2 male data set accounts for 31.7% of the difference in peak LODs between the sexes. Therefore, differences in sex-specific effects of *hg* alone cannot account for the observed differences in LOD scores between the sexes. However, there may have been sex differences in epistasis and/or allelic and dominance effects of growth QTLs located elsewhere on the genome or differences in the number of growth QTLs between the sexes. If such differences produced larger effects in the males, this could have increased the genetic "noise" and contributed in lowering the *hg* peak LOD score in F_2 males.

Our F_2 females and F_2 males differed in genetic composition of sex chromosomes. An F_2 female from the intercross of an HG (grand dam) × CAST (grand sire) carried on average 50% HG/HG and 50% HG/CAST loci on chromosome *X*. In contrast, an F_2 male was hemizygous on average for 50% HG and 50% CAST chromosome *X* alleles. Recent studies have provided evidence for the existence of growth QTLs on the *X* chromosome in mice (HASTINGS and VEERKAMP 1993; VEERKAMP *et al.* 1993; RANCE *et al.* 1994). Because the genetic composition of the *X* chromosomes was different between the F_2 females and F_2 males, *X*-linked growth QTLs could have produced different effects between the sexes; this may have contributed to the difference in the *hg* peak LOD scores between F_2 males and F_2 females.

Based on the data set studied here, we cannot differentiate which of the aforementioned possibilities or their combinations were the sources for the difference

in the *hg* peak LOD scores between the sexes. A genome-wide mapping and the analysis of genetic factors controlling postweaning gain in females and males of this intercross may provide such answers.

Transmission ratio analysis: Our analysis revealed that allelic and genotypic transmission ratios of most of the chromosome *10* markers were significantly altered in F_2 males but not in F_2 females. Several studies involving backcrosses between inbred strains of *M. m. domesticus* and a strain of wild mice *M. spretus* (JUSTICE *et al.* 1990; ROWE *et al.* 1994) or *M. m. musculus* (KOZAK *et al.* 1990) have also detected segregation distortion in the region of the mouse chromosome *10* studied here, but there were no reports on differences in allelic inheritance between sexes. Sex-specific differences in allelic inheritance of a region from chromosome *2* were demonstrated in a study of backcross mice originating from a cross between (C57BL/6J × *M. spretus*) F_1 × C57BL/6J (SIRACUSA *et al.* 1991). The authors provided elaborate discussion on potential sources of distortion in allelic inheritance and possible explanations for the observed sex-specific differences. They suggested that transmission distortion may be due to differences in production or survival of gametes (*e.g.*, during gametogenesis in F_1 female and F_1 male parents in our case), differences in fertilization efficiencies and differences in the survival of embryos or neonatal progeny.

The main factor contributing to the transmission distortion in F_2 males in our study was underrepresentation of F_2 males that were homozygous for CAST alleles at chromosome *10* loci. The mortality of neonatal progeny (between birth and ~10 weeks of age) was low in our cross. From a total of 403 F_2 mice, 5 pups died between birth and 2 weeks of age, while 1 male (HG/HG for the analyzed chromosome *10* markers) and 1 female died between 3 and 4 weeks of age. Therefore, the neonatal loss was too small to account for the observed transmission distortion in F_2 males.

It is likely that transmission distortion in F_2 males occurred in prenatal stages, during gametogenesis, fertilization or embryogenesis. One possible explanation would be that homozygosity for CAST alleles at a locus (loci), from the analyzed region of chromosome *10* would have an adverse effect on survival of male embryos or that male embryos that were heterozygous or homozygous for HG alleles at a locus (loci) from the analyzed region of chromosome *10* would have selective survival advantage. Alternatively, it may be that HG × CAST F_1 female's reproductive environment might have been hostile to male embryos homozygous for CAST alleles at a chromosome *10* locus (loci) or might have provided selective advantage to male embryos that were heterozygous or homozygous for HG alleles at a chromosome *10* locus (loci). If the aforementioned effects during embryogenesis were the source of transmission distortion, they should be expected to result in a significant difference in the ratio of neonatal females:males (*e.g.*, fewer male mice). However, sex ratios in our experiment (213 females:190 males) did not differ significantly from a Mendelian segregation ratio of 1:1 ($P < 0.05$). It is then less likely that the loss of male embryos carrying CAST alleles from the chromosome *10* region analyzed here or selection for embryos that were heterozygous or homozygous for HG alleles at a locus (loci) of chromosome *10* is the source of transmission distortion observed in F_2 males.

Another explanation for transmission distortion in F_2 males would be the differences in production, survival and/or fertilization efficiency between the *Y* chromosome-bearing sperm carrying CAST alleles at a chromosome *10* locus (loci) and the *Y* chromosome-bearing sperm carrying HG alleles at a chromosome *10* locus (loci). Either type of sperm may be selected for or against. Differences in production or survival of the two types of *Y* chromosome-bearing sperm could be tested by genotyping single sperm of an HG × CAST F_1 male for chromosome *10* and *Y*-chromosome loci (SCHMITT *et al.* 1994). This would allow determining a ratio of the two types of *Y* chromosome-bearing sperm and testing of this ratio for significant deviations from the expected 1:1 ratio. If this ratio is not significantly different from the expected, one would then test for differences in fertilization efficiency of the two types of *Y* chromosome-bearing sperm of an HG × CAST F_1 male. The test would involve sexing and genotyping for PCR-based markers of male preimplantation embryos (HORVAT *et al.* 1993) to analyze for chromosome *10* marker segregation. If the male preimplantation embryos carrying CAST alleles at a chromosome *10* locus (loci) are underrepresented, this may suggest that the difference in fertilization efficiency between the *Y* chromosome-bearing sperm carrying CAST alleles at a locus (loci) from the analyzed region of chromosome *10* and the *Y* chromosome-bearing sperm carrying HG alleles at a locus (loci) of chromosome *10* may account for the observed transmission distortion in F_2 males.

Improving the resolution of the *hg* map position in the present population: Although we defined a fairly small genomic segment containing *hg*, the resolution of the present *hg* genetic map is not sufficient for the application of physical mapping and map-based cloning, which require subcentiMorgan resolution. Some increase in the map resolution of *hg* may be achieved using the mapping population employed in this study. Including more markers around *hg* in the interval mapping analysis may decrease the *hg* LOD 2 support interval. We examined this empirically by performing interval analysis with markers at various levels of density. For example, in F_2 females, an analysis involving markers at ~10-cM spacing (*D10Mit31*, *Igf1*, *D10Mit12*, *D10Mit14*) produced a LOD 2 support interval of 12 cM. The addition of *D10Mit42*, *D10Mit41*, *D10Nds2*, *D10Mit9* and *D10Mit10* resulted in narrowing the LOD 2 support interval to 4.2 cM. Furthermore, adding the

Dcn marker, which was typed only in 17 F_2 females recombinant in the interval *D10Mit41* to *D10Mit12*, still resulted in decreased size of the LOD 2 support interval to 3.7 cM. Therefore, reanalyzing our data set with additional markers closely linked to *hg* may further narrow the *hg* LOD 2 support interval and hence potentially increase the accuracy of *hg* mapping. However, as the number of recombinants becomes limiting, adding more markers will not increase the map resolution. DARVASI *et al.* (1993) demonstrated that even for a QTL of large effect (similar to *hg*), in a large backcross population ($n = 1000$), and using an infinite number of markers, a resolution of the QTL map location needed for physical mapping (~1 cM) can not be achieved. Although the resolution of the *hg* map location resulted in some improvement as we added more markers, the study of DARVASI *et al.* (1993) supports our suggestion that further increase in marker density in our cross may not appreciably refine the map resolution of *hg*.

Another way to improve the accuracy of the *hg* map location using the mapping population employed here would be to detect other QTLs controlling postweaning gain located elsewhere on the genome and include this information in the mapping of *hg*. Recently, ZENG (1994) described a methodology combining interval mapping with multiple regression, which involves multiple QTL models to increase the QTL mapping accuracy. Following the same objective, JANSEN and STAM (1994) presented a similar approach, which entails multiple QTL models and the use of parental and F_1 phenotypic data. Applying these approaches to *hg* mapping, an experiment would involve screening of the present F_2 population for more markers covering the rest of the chromosome *10* and other chromosomes and reanalyzing our data set with the methodologies described above. This analysis may help to reduce the interfering effects of other (especially linked) QTLs, decrease the unexplained "noise", and potentially increase the precision of *hg* mapping. In addition, it may uncover other QTLs controlling postweaning gain and possible modifiers of *hg*.

Future fine resolution mapping of *hg*: Although using a denser spacing of markers or employing multiple QTL model analysis in the present cross may improve the resolution of the *hg* map, it will not result in a subcentiMorgan map of the region containing *hg*. The size and the nature of the cross (intersubspecific) and the mapping methodology itself has its limitations for providing a very fine genetic map resolution of a QTL (MICHELMORE and SHAW 1988). Ideally, one would proceed with genetic characterization of the region containing *hg* with the approach used by SHRIMPTON and ROBERTSON (1988) in mapping of the linked bristle polygenes in *Drosophila melanogaster*. This approach is based on an analysis of several congenic lines recombinant within a small targeted region to fine map QTL(s) in this region and to test for epistatic interactions between tightly linked QTLs. Although fine mapping of *hg* using the approach of SHRIMPTON and ROBERTSON (1988) is theoretically feasible in mice, it would not be practical. The development of several congenic lines carrying small genomic segments from the region containing *hg* would be a time consuming and costly process.

The fine mapping of *hg* would be more feasible using crosses of congenic strains (PATERSON *et al.* 1990; JACOB *et al.* 1991; LANDER and SCHORK 1994). A congenic strain C57BL/6J-*hghg* is already available, and could be crossed to C57BL/6J to produce a mapping population. Assuming that our congenic pair is genetically identical in regions unlinked to *hg*, then the genetic noise due to segregation of unlinked QTLs would be eliminated and a higher resolution genetic map of *hg* could be obtained. Fine mapping of *hg* would be based on recombinants in the interval containing *hg* (between *D10Mit9* and *Dcn*) and progeny tests of these recombinants. The ability to define small genetic intervals will depend in part on the density of available genetic markers. The genetic divergence of the region containing *hg* in this congenic pair appears to be high, because 10 of 18 currently available microsatellite markers between *D10Mit9* and *D10Mit12* are polymorphic. New microsatellite markers are continually being produced in mice and will most likely provide additional markers in the region around *hg*. New markers in the targeted *hg* genomic region can also be developed using approaches based on the random amplified polymorphic DNA (GIOVANNONI *et al.* 1991; MICHELMORE *et al.* 1991; HORVAT and MEDRANO 1994), the amplified fragment length polymorphism assay (M. ZABEAU, Keygene personal communication), or representational difference analysis (LISITSYN *et al.* 1994).

Another important issue in future fine mapping of *hg* will be our ability to unambiguously discriminate between the *hghg* and the non-*hghg* genotypes based on phenotype. Although the *hg* locus produces a major increase in the postweaning gain in homozygote individuals, it is not possible to clearly separate all *hghg* from non-*hghg* mice in segregating populations. This was evident in several crosses involving high-growth mice and in backcrosses during the development of the HG line. Therefore, we can expect some overlap in the growth distribution curves between *hghg* and non-*hghg* mice in our future fine mapping crosses involving congenic strains. To overcome this problem, mapping using mice from extremes of the growth distribution curve and progeny testing of mice in the overlap of the growth distribution curves will be required. Alternatively, we can search for a trait resulting from the *hg* action that will result in a clearer separation of *hghg* and non-*hghg* mice. Recently, SUMMERS and MEDRANO (1994) found that HG mice have a significantly higher number of muscle fibers in the *soleus* muscle compared with

C57BL/6J. If the high fiber number/muscle cosegregates with *hg* and can clearly separate *hg* genotypes in crosses, this trait may provide a valuable parameter in future high resolution genetic mapping of *hg*.

Application of mapping *hg*: A denser map of genetic markers around the *hg* locus and more accurate characterization of the high-growth phenotype should enable fine *hg* mapping in crosses of congenic strains. The aim of fine mapping is to narrow the *hg* candidate region to a small genomic segment suitable for physical mapping and cloning of *hg*. Cloning of *hg* will allow molecular characterization of *hg* product and promote further functional studies to examine the role of *hg* in the control of mammalian growth. Furthermore, knowledge of *hg* and/or markers linked to *hg* will also permit studies of homologous regions in other species. For instance, the knowledge that *Dcn* is closely linked to *hg* may be applied to domestic animals. The cDNAs of *Dcn* have already been sequence characterized in cattle (DAY *et al.* 1987) and chickens (LI *et al.* 1992). Therefore, PCR-based markers in this gene can be developed in these and, potentially, also in related species. These markers can then be tested for associations with growth traits in domestic animals and may uncover a QTL with positive effects on growth. Such markers could provide efficient tools for identifying animals with a high genetic potential for growth.

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